

Roles of sensory nerves in the regulation of radiation-induced structural and functional changes in the heart

Supplementary Methods

Echocardiography

At 1, 3, and 6 months after irradiation, echocardiography was performed with a Vevo 2100 imaging system and MS250 transducer (13-24 MHz) (VisualSonics, Toronto, Canada). Animals were anesthetized with 2% isoflurane inhalation and hair was removed from the chest with clippers followed by a depilatory cream. The rats were placed on a warmed platform that recorded ECGs from the paws. ECGs were used to detect bradycardia (heart rate <250 bpm) and arrhythmia. Long axis B-mode recordings were used to determine the size of the aortic outflow tract. Short axis M-mode recordings at the mid left ventricular (LV) level were used to obtain LV mass, LV anterior wall (LVAW), LV posterior wall (LVPW), LV inner diameter (LVID), ejection fraction (EF), fractional shortening (FS), stroke volume, and cardiac output.

Histology and immunohistochemistry

Animals were anesthetized with 3% isoflurane inhalation, and hearts were isolated, briefly rinsed via retrograde perfusion, fixed in 10% buffered formalin or methanol Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid) and embedded in paraffin. Longitudinal sections of 5 μ m were used for histology or immunohistochemistry.

For determination of collagen deposition formalin-fixed sections were rehydrated and incubated in Sirius Red (American MasterTech, Lodi, CA) supplemented with Fast Green (Fisher Scientific, Waltham, MA) for 1 hour, followed by 0.5% acetic acid for 14 min.

For determination of mast cell numbers, formalin-fixed sections were rehydrated and incubated in 0.5% Toluidine Blue in 0.5 N HCl for 72 hours, followed by 0.7 N HCl for 10 min.

For determination of apoptotic nuclei, formalin-fixed sections were rehydrated and stained with the CardioTACS™ Kit (Trevigen, Gaithersburg, MD), according to the manufacturer's instructions.

For immunohistochemical analysis of CD2 (to indicate T-cells and natural killer cells), CD68 (monocytes and macrophages), and von Willebrand factor (vWf, a prothrombotic and profibrogenic marker for endothelial injury), methanol Carnoy's-fixed sections were rehydrated and incubated with 1% H₂O₂ in methanol to block endogenous peroxidase, followed by 10% normal serum in 3% dry powdered milk and 0.2% BSA to block non-specific antibody binding. Sections were incubated overnight with mouse anti-CD2 (1:100, Cedarlane Laboratories, Burlington, NC), mouse anti-CD68 (1:100, Abcam, Cambridge, UK), or rabbit anti-vWf (1:800, Dako, Glostrup, Denmark), followed by goat anti-rabbit IgG (1:400, Vector Laboratories, Burlingame, CA) or donkey anti-mouse IgG (1:400, Fitzgerald Industries, North Acton, MA) and an avidin-biotin-peroxidase complex (Vector Laboratories) for 45 minutes. Bound antibodies were visualized with 0.5 mg/mL DAB (Sigma-Aldrich).

The sections stained with Sirius Red / Fast Green were scanned with a ScanScope CS2 slide scanner and analyzed with ImageScope 12 software (Aperio, Vista, CA). To determine deposition of interstitial collagen, all perivascular staining was excluded from analysis. The percentage area positive for interstitial collagen was determined as: $100 \times \frac{\text{area of Sirius Red}}{\text{area of Sirius Red} + \text{area of Fast Green}}$. All other sections were examined with an Axioskop transmitted light microscope (Carl Zeiss, Oberkochen, Germany). All mast cells, CD2-positive cells, CD68-positive cells, and apoptotic nuclei in the left and right ventricles were counted. Sections stained for vWf were blindly scored for areas occupied by vWf-positive microvessels on a scale from 0 to 3.

Western-Blots

Animals were anesthetized with 3% isoflurane inhalation, and LV tissue was isolated, snap-frozen and stored at -80°C. LV tissue was homogenized in RIPA buffer with a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). A total of 60 µg protein was prepared in Laemmli buffer containing 1:20 β-mercaptoethanol and boiled for 2-3 minutes, separated in 4-20% gradient polyacrylamide gels and transferred to PVDF membranes (Bio-Rad). After blocking in 5% non-fat dry milk, membranes were incubated with goat anti-CGRP (1:1,000, Abcam), rabbit anti-nuclear receptor subfamily 4, group A (NR4A) 1 and 2 (NR4A1/2, 1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-microtubule associated protein 1 light chain 3A (LC3A, 1:2,000 Cell Signaling Technology, Danvers, MA), or rabbit anti-mast cell tryptase (1:50,000 Santa Cruz). Protein loading was corrected with mouse anti-GAPDH (1:20,000 Santa Cruz Biotechnology). Primary antibody incubations were followed by HRP-conjugated mouse anti-goat (1:15,000, Santa Cruz), goat anti-mouse (1:5,000 Jackson ImmunoResearch), or mouse anti-rabbit (1:4,000 Cell Signaling Technology). Antibody binding was visualized with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA) and CL-Xposure films (Thermo Fisher Scientific). Films were scanned with an AlphaImager HP System (ProteinSimple, Santa Clara, CA), and density of protein bands was quantified with ImageJ software.